

THE EFFECTS OF METABOLITES OF POLYPHENOLS AND VITAMIN E
ON MODULATING THE HIGH GLUCOSE-INDUCED
ENDOTHELIAL DYSFUNCTION

by
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ABSTRACT

Hyperglycemia is an obvious candidate contributor in the development of endothelial dysfunction in association with insulin resistance. A great deal of evidence demonstrates that polyphenols and vitamin E can improve endothelial dysfunction, but little research has examined whether their metabolites exert protective effects on endothelial cells. In the current study, we determined the mechanisms by which metabolites can protect against high glucose-induced endothelial dysfunction in human aortic endothelial cells (HAECs). Confluent HAECs were treated with 5mM or 25mM glucose for 48h, or pretreated with 3-hydroxyphenylpropionic acid (3-HPP) at 1 μ M, Piceatannol (Picea) at 5 μ M, quercetin 3-glucuronide (Quer-3-glu) at 2 μ M, and 2,7,8-trimethyl-2-(beta-carboxyethyl)-6-hydroxychroman (γ -CEHC) at 3 μ M, respectively, for 24h, and then treated with high glucose (25mM) for 48h. Nitric oxide (NO) production, total and phosphorylation of endothelial NO synthase (eNOS), protein kinase B (Akt) and extracellular signal regulated kinase (ERK), and reactive oxygen species (ROS)/reactive nitrogen species (RNS) production were determined. In HAECs, insulin-stimulated NO production (59%), eNOS^{Ser1177}, and Akt^{Ser473} phosphorylation (1.3-fold and 1.5-fold, respectively) was increased ($p < 0.05$) in cells treated with 5mM glucose. Insulin had no stimulatory effect on NO or eNOS signaling in cells treated with 25mM glucose. However, cells pretreated for 24h with 3-HPP (1 μ M), Picea (5 μ M), Quer-3-glu (2 μ M), and γ -CEHC (3 μ M), respectively, and then treated with 25mM glucose for 48h, had

normalized ($p < 0.05$) insulin-stimulated NO production, eNOS^{Ser1177}, and Akt^{Ser473} phosphorylation. There was no difference in basal NO production, eNOS^{Ser117}, AktSer⁴⁷³, ERK^{Thr202/204} phosphorylation, or insulin-stimulated ERK^{Thr202/204} phosphorylation at each treatment. ROS/RNS production was stimulated by treatment with 25mM glucose (65%, $p < 0.05$), and prevented when HAECs were pretreated with metabolites. In conclusion, metabolites of quercetin, resveratrol, catechin, and gamma tocopherol rescue high glucose-induced endothelial dysfunction as evidenced by increased NO production. We propose the mechanism occurred through a reduction of ROS/RNS that normalized Akt-mediated eNOS^{Ser1177} phosphorylation.

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INTRODUCTION

Endothelial dysfunction is a systemic pathological state of the endothelium and can be broadly defined as an imbalance between vasodilating mediators (such as NO, endothelium-derived hyperpolarizing factor (EDHF) and prostacylin) and vasoconstricting mediators (such as endothelin-1, thromboxane A₂ and reactive oxygen species (ROS)) produced by the endothelial cells (1). It is known that endothelial dysfunction is commonly associated with diminished NO bioavailability, which is due to impaired NO production by the endothelium and/or increased inactivation of NO by ROS. Moreover, current literature indicates that the reduced endothelium-derived NO, produced by endothelial nitric-oxide synthase (eNOS), has been identified as a hallmark in endothelial dysfunction and the most relevant mechanism in many cardiovascular dysfunctions (2). In addition, decreased NO bioavailability is well described in patients with type 1 diabetes (3) and type 2 diabetes (4) and also in cardiovascular disorders such as hypertension and atherosclerosis associated with insulin resistance (5).

Diabetic vascular dysfunction is a major clinical problem that predisposes patients to a variety of cardiovascular diseases. High glucose (or hyperglycemia) is an important causal factor in the development of endothelial dysfunction in diabetes. Many studies have indicated that high glucose-induced endothelial dysfunction may be due to increased ROS production (6-8). For example, a report has suggested that acute (15–30 min) stimulation with high glucose inhibits insulin-stimulated NO production as a result of

reduced eNOS^{Ser1177} phosphorylation in human umbilical vein endothelial cells (HUVECs) (9). The overproduction of ROS can reduce endothelial NO availability via different pathways such as formation of peroxynitrite (ONOO⁻) from NO by superoxide. Reduction in NOS enzyme expression and activity can also occur by increasing asymmetric dimethylarginine (ADMA) levels, as well as eNOS uncoupling due to increased BH₄ oxidation to BH₂ (7, 10-14).

Many studies have shown that nutritional supplements, such as grape seed extract, resveratrol, quercetin, and vitamin E, can prevent endothelial dysfunction through different mechanisms. Recent studies have suggested that grape seed extract has many beneficial effects on the cardiovascular system (15), such as reducing inflammatory stress (16), preventing high glucose induced impairment of eNOS activity by improving eNOS phosphorylation (17), decreasing plasma cholesterol and atherosclerosis (18), lowering arterial blood pressure (19), increasing endothelium-dependent relaxation of aortic rings (20), as well as improving coronary and aortic flow (21). In addition, the effects of resveratrol and quercetin on modulating endothelial function have been widely studied. The improvement of endothelial function by these two components was largely attributed to enhance endothelial NO bioactivity through multiple mechanisms (22-24), such as enhancing eNOS expression (25), phosphorylation of eNOS at Ser¹¹⁷⁷, AMPK at Thr¹⁷² (26) and BH₄ biosynthesis (27), decreasing endogenous eNOS inhibitor (28), scavenging superoxide anion (29, 30), inhibiting xanthine oxidase and NADPH oxidase (31), as well as increasing intracellular Ca²⁺ concentration (32). Studies also have demonstrated that resveratrol and quercetin can lower blood pressure in hypertensive humans and animals, though a mechanism in humans has not yet to be defined (33-36). The cardioprotective

effects are not limited to polyphenols either since many clinical and animal studies have showed that gamma tocopherol (γ -T), the major form of vitamin E consumed in the US diet, has protective effects on the endothelium. Previous work has shown enhanced endothelium-dependent hyperemia-induced vasodilatation of brachial artery (37), increased NO production and platelet eNOS activity (38), decreased ADP-induced platelet aggregation (39), lipid peroxidation, and disruption in NO homeostasis [Mah & Bruno, unpublished data, 2012].

However, the bioavailability of these nutritional supplements is complicated. After ingestion, these supplements are quickly digested and absorbed. The dominant forms that exist in blood are their physiologic metabolites. Human studies showed that supplementation with grape seed polyphenols (1000mg/d) result in increased urinary excretion of 3- hydroxyphenylpropionic acid (3-HPP) (0.2 μ M), suggesting that this compound is a major phenolic acid breakdown product in vivo (40). Similarly, γ -T supplementation (500mg/d) increases plasma γ -carboxyethyl- hydroxychroman (γ -CEHC) (3 μ M) concentration by > 9-fold [Mah & Bruno, unpublished data, 2012]. Other human and animal studies found that piceatannol derived from resveratrol (300 ml of red wine/d) is the major metabolite found in liver microsome and blood serum (5 μ M) (41, 42). In addition, a human study demonstrated that quercetin 3-glucuronide (Quer-3-glu) (2 μ M) presents in plasma of human subjects 1.5 h after consumption of onions (100 mg/d-330 μ mol quercetin equivalent) (43). In spite of studies such as the aforementioned ones, many previous in vitro studies that have been done to elucidate mechanisms of endothelial cell protection have used the parent compounds, not their metabolites. Therefore, the purpose of this study is to determine the mechanisms by which metabolites

of grape seed extract, resveratrol, quercetin, and vitamin E, can protect against high glucose-induced endothelial dysfunction.

MATERIALS AND METHODS

Materials

Human aortic endothelial cells (HAECs) were purchased commercially (Lonza, Carlsbad, California). 3-hydroxyphenylpropionic acid (3-HPP) and quercetin 3-glucuronide (Quer-3-glu) were purchased from Sigma Chemical (St. Louis, Missouri, USA). Piceatannol (Picea) was purchased from A. G. Scientific Inc. (San Diego, CA, USA). α -CEHC was purchased from Encore Pharmaceuticals (Riverside, CA, USA). Nitrate/Nitrite colorimetric assay kit was purchased from Cayman Chemical (Ann Arbor, MI). OxiSelect™ in vitro ROS/RNS assay kit was purchased from Cell Biolab, Inc. (San Diego, CA, USA). Antibodies directed against p-eNOS^{Ser1177}, total eNOS, p-Akt^{Ser473}, total Akt, p-ERK^{Thr 202/204}, total ERK, and actin were purchased from Cell Signal Technology (Beverly, MA). All of the other reagents will be purchased from Sigma Chemical except special statement.

Cell culture

HAECs were cultured in T-75 in Medium M199 (GIBCO, CA) containing 2% FBS and endothelial growth supplements EGM-2 (Life Technologies, CA) in a humidified atmosphere (5% CO₂ / 95% O₂, 37°C) and passaged when at 80% confluence. The cells used for experiments were 70-80% confluent and between passage 3 and passage 6.

When HAECs reached 80% confluence, the media in the T75 flask were aspirated and removed. 2ml of 0.05% trypsin (Cellgro, Mediatech Inc., Manassas, Virginia) were administered to the cells. The cells were then incubated for 2-3 minutes at 37°C and viewed under a microscope to ensure all were dislodged from the flask surface. Proper quantity of Medium 199 supplemented with 2% FBS and endothelial growth supplements EGM-2 were added to the trypsinized cells and a pipette was used to wash the bottom of the flask in order to ensure all cells were dislodged and distributed evenly throughout the medium. The cells were then seeded to the appropriate sterile petri dishes for experiments and a new T-75 flask for further cell culture. After adding the medium, each container was placed in an incubator and cells were grown in a humidified atmosphere of 5% CO₂ / 95% O₂ at 37°C.

Cell treatment

Before the treatment, the cells were incubated overnight in the medium without serum. Then, one group of cells was treated with 5mM glucose supplemented with 20mM mannitol to maintain osmolarity (vehicle control) for 48h; one group of cells was treated with 25mM glucose (positive control) for 48h; the other four groups of cells were pre-treated with the metabolites (1μM 3-HPP, 5μM Picea, 2μM Quer-3-glu, and 3μM α -CEHC), respectively, for 24h, and then treated with 25mM glucose for 48h. In the last 10 minutes, the cells were treated with or without insulin (100nM). Because metabolites were dissolved in dimethyl sulfoxide (DMSO, Mallinckrodt, Phillipsburg, New Jersey), vehicle control group and positive control group were added the same quantity of DMSO for each treatment.

NO production

HAECs were grown and treated as described earlier. After treatment, the media were discarded. The cells were gently washed with iced PBS twice and then collected for NO assay. NO was rapidly oxidized to the stable nitrate (NO_2^-) and nitrite (NO_3^-), and NO production was evaluated by measuring the sum concentration of NO_2^- and NO_3^- (NO_x) in the cells. Briefly, the collected cells were treated with nitrate reductase to reduce nitrate to nitrite, which reacts with the Griess reagent to yield diazochromophore. Each sample (80 μL), or nitrate standard, containing 10 μL nitrate reductase and 10 μL nitrate reductase cofactors was incubated for 3h at 37 °C. At the end of the incubation period, 50 μL of Griess reagent R1 and 50 μL of Griess reagent R2 were added and incubated for 10 minutes. Total NO_x was quantified using the absorbance at 550 nm compared with the absorbance of identically prepared nitrite standards. Absorbance data were converted into concentrations based on standard curves constructed with sodium nitrate and normalized to protein concentration of the samples.

Detection of ROS/RNS

HAECs were grown and treated as described earlier, but the cells were not treated with insulin. After treatment, the media were discarded. The cells were gently washed with iced PBS for three times and then collected for ROS/RNS assay. The dichlorodihydrofluorescein DiOxyQ (DCFH-DiOxyQ), which is based on similar chemistry to the 2', 7'- dichlorodihydrofluorescein diacetate, was used to assess ROS/RNS generation. Briefly, the DCFH-DiOxyQ probe was first primed with a quench removal reagent, and subsequently stabilized in the highly reactive DCFH form. When

the collected cells were treated with DCFH, ROS/RNS species reacted with DCFH, which was rapidly oxidized to highly fluorescent 2', 7'- dichlorodihydrofluorescein (DCF). DCF standard was prepared before the measurement. Each sample (50 μ L) was incubated with 50 μ L catalyst for 5 minutes at 37 $^{\circ}$ C , and then incubated with 100 μ L DCFH for 15-45 minutes at 37 $^{\circ}$ C in dark ambiance. Relative fluorescence was measured using a plate reader (Molecular Devices, Silicon Valley, CA) at excitation and emission wavelengths of 480 and 530 nm, respectively. Fluorescence data were converted into concentrations based on DCF standard curves and normalized to protein concentration of the samples.

Western blotting

After 48h treatment, HAECs were washed with PBS three times, then lysed using cell lysis buffer (in mM, 150 NaCl, 20 Tris-HCl, 2 EDTA, 50 NaF, 10 HEPES, 50 Na₂H₂P₂O₇, and 1% Triton X-100). Protein concentrations of cells were determined by a protein assay using bovine serum albumin (BSA) standard (BioRad, Hercules, CA). Briefly, the 40 μ g protein loaded in each well was subjected to electrophoresis in 10% SDS polyacrylamide gel. Proteins in gels were transferred to PVDF membranes then blocked with 5% nonfat milk in TBS-T. After blocking, membranes were incubated overnight at 4 $^{\circ}$ C with primary antibodies in 1:1000 dilutions of 5% BSA in Tris buffer with 0.05% Tween-20 (phospho specific antibodies) or 5% nonfat milk in TBS-T (non-phospho specific antibodies). After washing, membranes were incubated for 1h with secondary antibodies conjugated to horseradish peroxidase (goat anti-rabbit, Cell Signal Technology, Beverly, MA) in 1:5000 dilutions. Signals were visualized by enhanced

chemiluminescence (Cell Signal Technology, Beverly, MA). After exposure, membranes were stripped and probed for actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as a loading control. Immunoblot band densities were quantified with a Kodak Gel Logic 1500 Imaging System and Kodak Molecular Imaging Software (Kodak MI v 4.0, Eastman Kodak Company, New Haven, CT).

Cell viability

Cell viability was assessed in HAECs. The numbers of attached cells were accounted in a fixed area before and after different treatments. The 0.25% trypsin was served as the positive control.

Statistical analyses

To prepare Western blot data for statistical analysis, a normalization procedure was conducted. 5mM glucose supplemented with 20mM mannitol without insulin stimulated (5mM Glu) served as the global control for normalization. For each protein assessed by Western blot, all results were normalized to controls of 5mM Glu (phospho : total) with those levels set at 1. Fold changes for other treatment conditions were calculated and expressed based on controls. Data were analyzed with one-way ANOVA using SPSS software and expressed as mean \pm SE of four to six independent experiments. When main effects were detected, Tukey post hoc tests were conducted to determine differences between conditions. $P < 0.05$ was considered significant.

RESULTS

Dose response experiments

The concentration of metabolites used in this study was based on previous literature (28, 40, 44) [Mah & Bruno, unpublished data, 2012]. A preliminary dose response experiment (3-HPP: 0.2, 1, 2 μ M; Picea: 5, 25, 50 μ M; Quer-3-glu: 2, 10, 20 μ M; γ -CEHC: 3, 15, 30 μ M, respectively) was conducted to determine the most efficient dose for the future study. Interestingly, the most efficient dose of all metabolites was similar to their physiological concentration in the blood, such as Picea at 5 μ M, Quer-3-glu at 2 μ M, and γ -CEHC at 3 μ M. The dose of 3-HPP is 5-fold greater than its physiological concentration.

3-HPP, Picea, Quer-3-glu, and γ -CEHC restores insulin-stimulated

NO production inhibited by high glucose in HAECs

To study the effects of high glucose, 3-HPP, Picea, Quer-3-glu, and γ -CEHC on insulin-stimulated NO production in, we examined the ability of insulin to stimulate NO synthesis in HAECs (Figure 1). As shown, insulin (100nM) significantly stimulated NO synthesis in HAECs treated with 5mM glucose for 48h (59% increased). In contrast, insulin had no significant effect on NO synthesis in HAECs treated with high glucose (25mM) for 48h. However, cells pretreated for 24h with 3-HPP (1 μ M), Picea (5 μ M), Quer-3-glu (2 μ M), and γ -CEHC (3 μ M), respectively, and then treated with high

glucose (25mM) for 48h, had normalized insulin-stimulated NO synthesis (Figure 1). There was no significant difference in basal NO synthesis at each treatment.

3-HPP, Picea, Quer-3-glu, and γ -CEHC restores insulin-stimulated
eNOS and Akt phosphorylation impaired
by high glucose in HAECs

To determine whether insulin-stimulated phosphorylation of eNOS, Akt, and ERK was impaired by high glucose and restored by 3-HPP, Picea, Quer-3-glu, and γ -CEHC, respectively, we determined the fold change of ratio of phosphorylated to total eNOS, Akt, and ERK in HAECs. Insulin-stimulated phosphorylation of eNOS at Ser¹¹⁷⁷ and Akt at Ser⁴⁷³ in HAECs treated with 5mM glucose for 48h (1.3-fold, $p < 0.05$ and 1.5-fold, $p < 0.05$, respectively) (Figure 2, 3). In contrast, insulin had no significant effect on phosphorylation of eNOS at Ser¹¹⁷⁷ and Akt at Ser⁴⁷³ in HAECs treated with high glucose (25mM) for 48h (Figure 2, 3). Cells treated with 3-HPP (1 μ M), Picea (5 μ M), Quer-3-glu (2 μ M), and γ -CEHC (3 μ M), respectively, before 25mM glucose challenge showed a normalization ($p < 0.05$) of insulin-stimulated phosphorylation of eNOS at Ser¹¹⁷⁷ and Akt at Ser⁴⁷³ to equivalent levels to that of 5mM glucose-incubated controls (Figure 2, 3). There was no significant difference in basal eNOS^{Ser117}, AktSer⁴⁷³, ERK^{Thr202/204} phosphorylation, or insulin-stimulated ERK^{Thr202/204} phosphorylation at each treatment (Figure 2, 3, and 4).

3-HPP, Picea, Quer-3-glu, and γ -CEHC inhibits high glucose-stimulated ROS/RNS production in HAECs

To determine whether high glucose-stimulated ROS/RNS production and the increased production was inhibited by 3-HPP, Picea, Quer-3-glu, and γ -CEHC, respectively, we assessed the total ROS/RNS production in HAECs (Figure 5). The ROS/RNS production was significantly increased in HAECs treated with high glucose (25mM) vs. 5mM glucose for 48h (65% increased). 24h pretreatment with 3-HPP (1 μ M), Picea (5 μ M), Quer-3-glu (2 μ M), and γ -CEHC (3 μ M), respectively, prevented the rise in ROS/RNS evoked by high glucose (25mM) (Figure 5) such that the total ROS/RNS production was similar to that of cells in 5mM glucose.

Cell viability is similar between treatments

We assessed cell viability in HAECs treated with 5mM glucose or high glucose (25mM) for 48h, or cells incubated with 25 mM glucose that were pretreated with 3-HPP (1 μ M), Picea (5 μ M), Quer-3-glu (2 μ M), and γ -CEHC (3 μ M) for 24h, respectively. There was no change of cell viability at each treatment; the cell death level was < 8% and it was not significant different as compared to control (Figure 6).

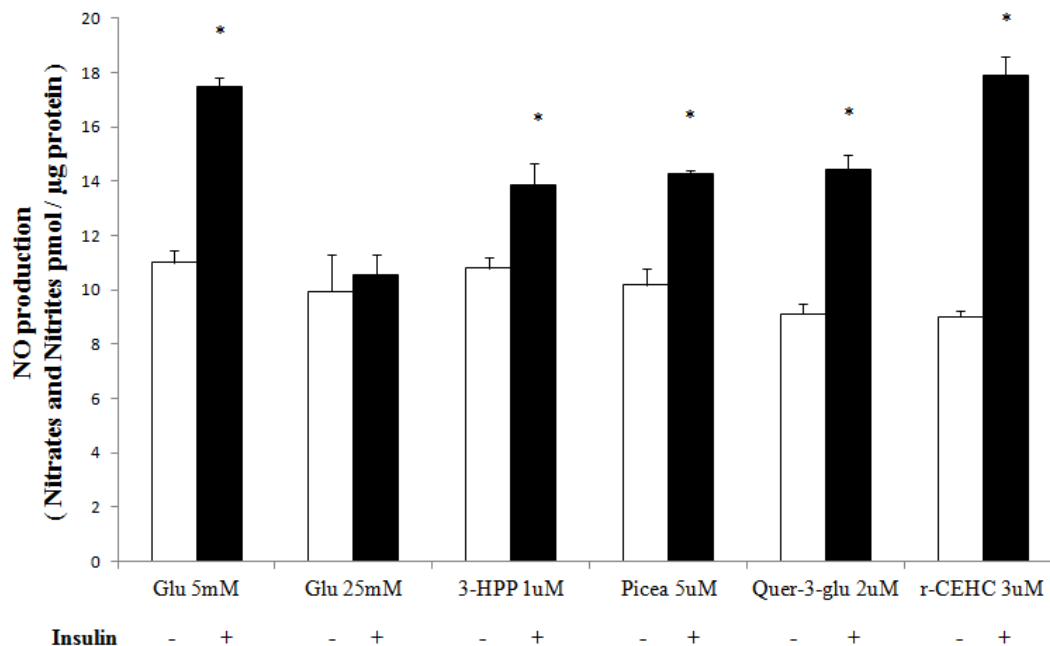


Figure 1. Effects of 5mM glucose, 25mM glucose, and metabolites, respectively, on insulin-stimulated NO production in HAECs. Confluent HAECs were treated with 5mM or 25mM glucose for 48h, or pretreated with 3-HPP (1μM), Picea (5μM), Quer-3-glu (2μM), and r-CEHC (3μM), respectively, for 24h, and then treated with high glucose (25mM) for 48h. Nitrate/nitrite (NO_x) production stimulated by insulin (100nM, 10mins) was measured using a colorimetric assay kit and normalized to protein content. Results represent mean ± SE from n = 4 separate experiments. * P < 0.05 vs. Glu 5mM (-) insulin.

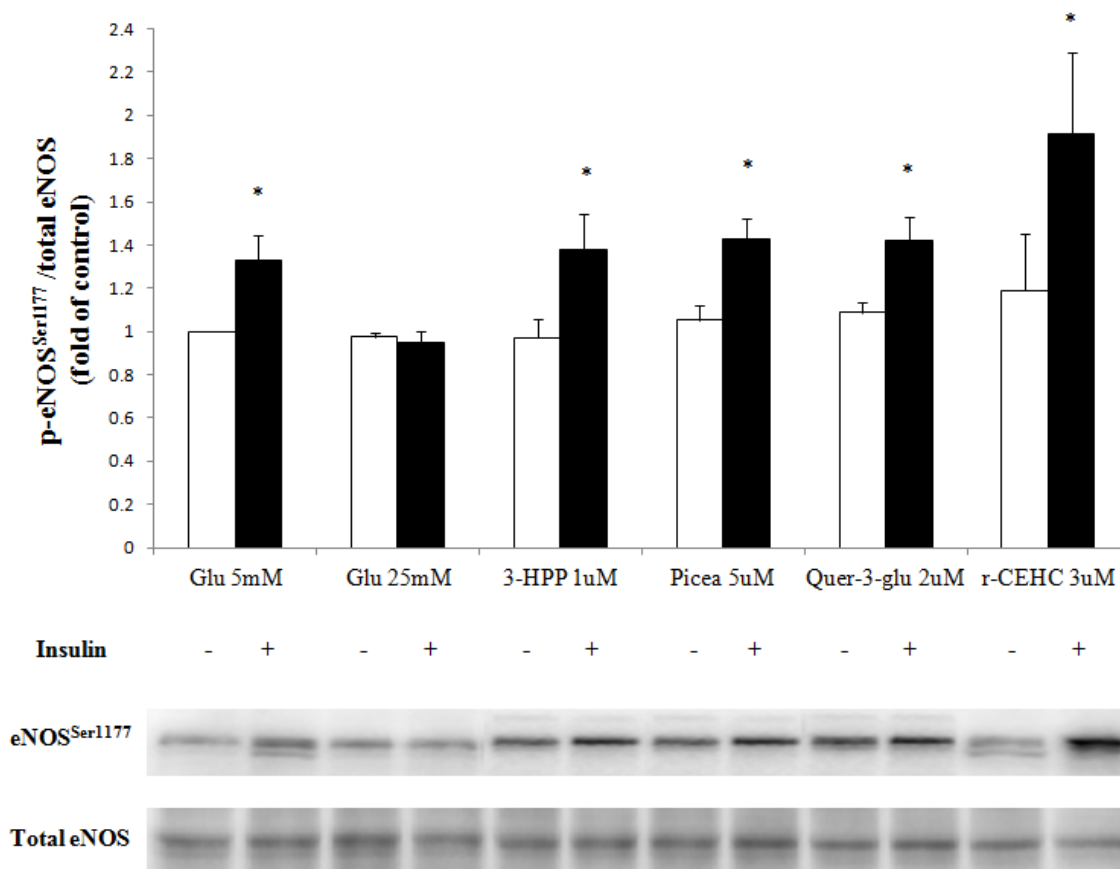


Figure 2. Effects of 5mM glucose, 25mM glucose, and metabolites, respectively, on insulin-stimulated eNOS phosphorylation (Ser1177) in HAECs. Confluent HAECs were treated with 5mM or 25mM glucose for 48h, or pretreated with 3-HPP (1 μ M), Picea (5 μ M), Quer-3-glu (2 μ M), and γ -CEHC (3 μ M), respectively, for 24h, and then treated with high glucose (25mM) for 48h. Total eNOS and p-eNOS^{Ser1177} protein levels in cell extracts were measured by Western blotting. The ratio of phosphorylated to total eNOS (mean \pm SE) of n = 6 separate experiments are expressed as fold change vs. Glu 5mM (-) insulin. * P < 0.05 vs. Glu 5mM (-) insulin.

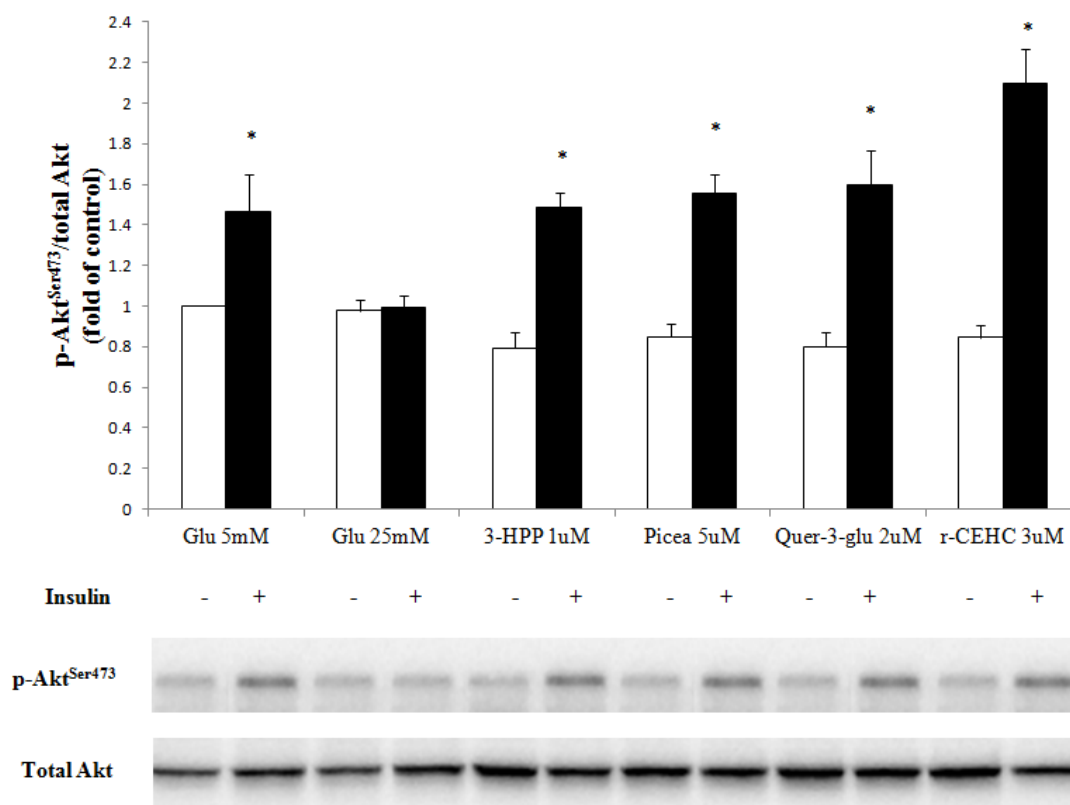


Figure 3. Effects of 5mM glucose, 25mM glucose, and metabolites, respectively, on insulin-stimulated Akt phosphorylation (Ser473) in HAECs. Confluent HAECs were treated with 5mM or 25mM glucose for 48h, or pretreated with 3-HPP (1μM), Picea (5μM), Quer-3-glu (2μM), and γ -CEHC (3μM), respectively, for 24h, and then treated with high glucose (25mM) for 48h. Total Akt and p-Akt^{Ser473} protein levels in cell extracts were measured by Western blotting. The ratio of phosphorylated to total Akt (mean \pm SE) of $n = 4$ separate experiments are expressed as fold change vs. Glu 5mM (-) insulin. * $P < 0.05$ vs. Glu 5mM (-) insulin.

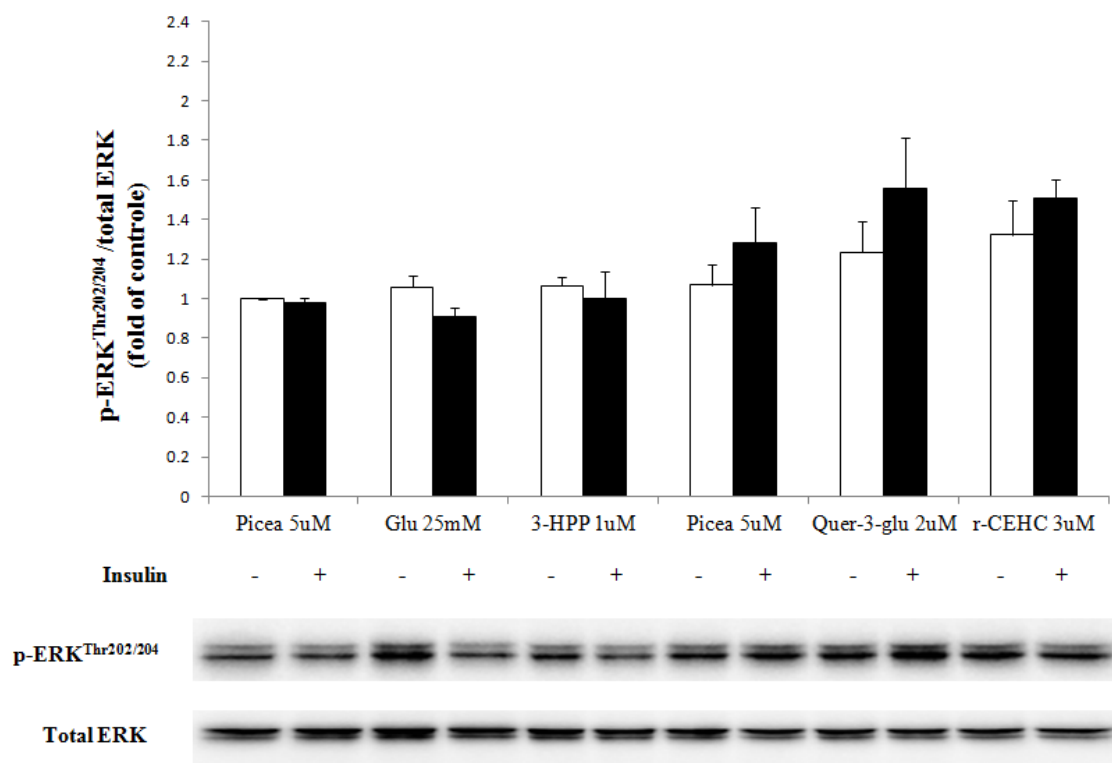


Figure 4. Effects of 5mM glucose, 25mM glucose, and metabolites, respectively, on insulin-stimulated ERK phosphorylation (Thr202/204) in HAECs. Confluent HAECs were treated with 5mM or 25mM glucose for 48h, or pretreated with 3-HPP (1μM), Picea (5μM), Quer-3-glu (2μM), and γ-CEHC (3μM), respectively, for 24h, and then treated with high glucose (25mM) for 48h. Total ERK and p-ERK^{Thr202/204} protein levels in cell extracts were measured by Western blotting. The ratio of phosphorylated to total ERK (mean ± SE) of n = 4 separate experiments are expressed as fold change vs. Glu 5mM (-) insulin. * P < 0.05 vs. Glu 5mM (-) insulin.

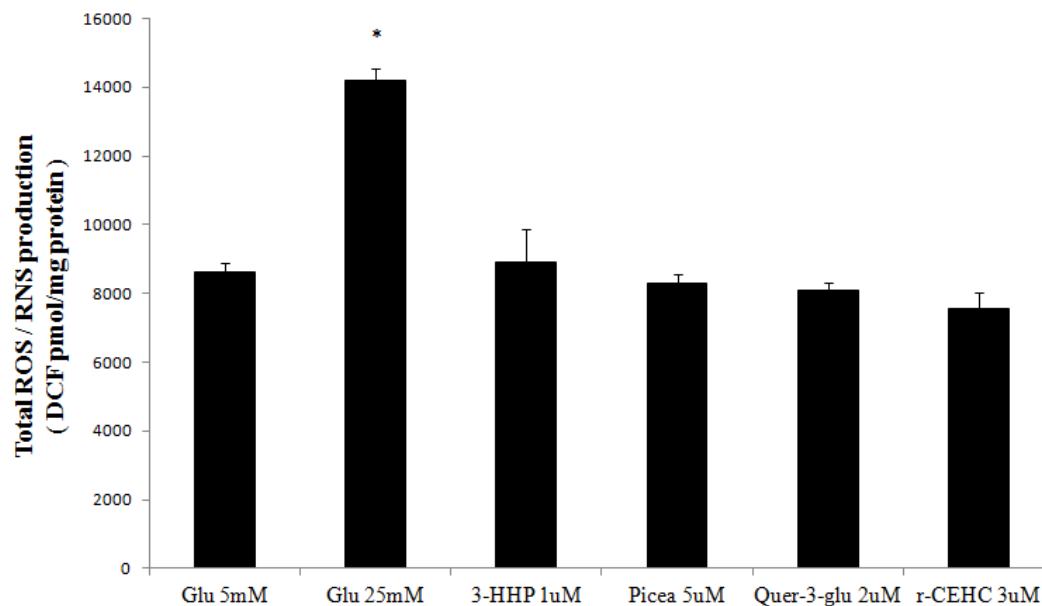


Figure 5. Effects of 5mM glucose, 25mM glucose, and metabolites, respectively, on total ROS/RNS production. Confluent HAECs were treated with 5mM or 25mM glucose for 48h, or pretreated with 3-HHP (1 μ M), Picea (5 μ M), Quer-3-glu (2 μ M), and γ -CEHC (3 μ M), respectively, for 24h, and then treated with high glucose (25mM) for 48h. DCF concentration was measured using a fluorescence assay kit and normalized to protein content. Results represent mean \pm SE from n = 4 separate experiments. * P < 0.05 vs. Glu 5mM (-) insulin.

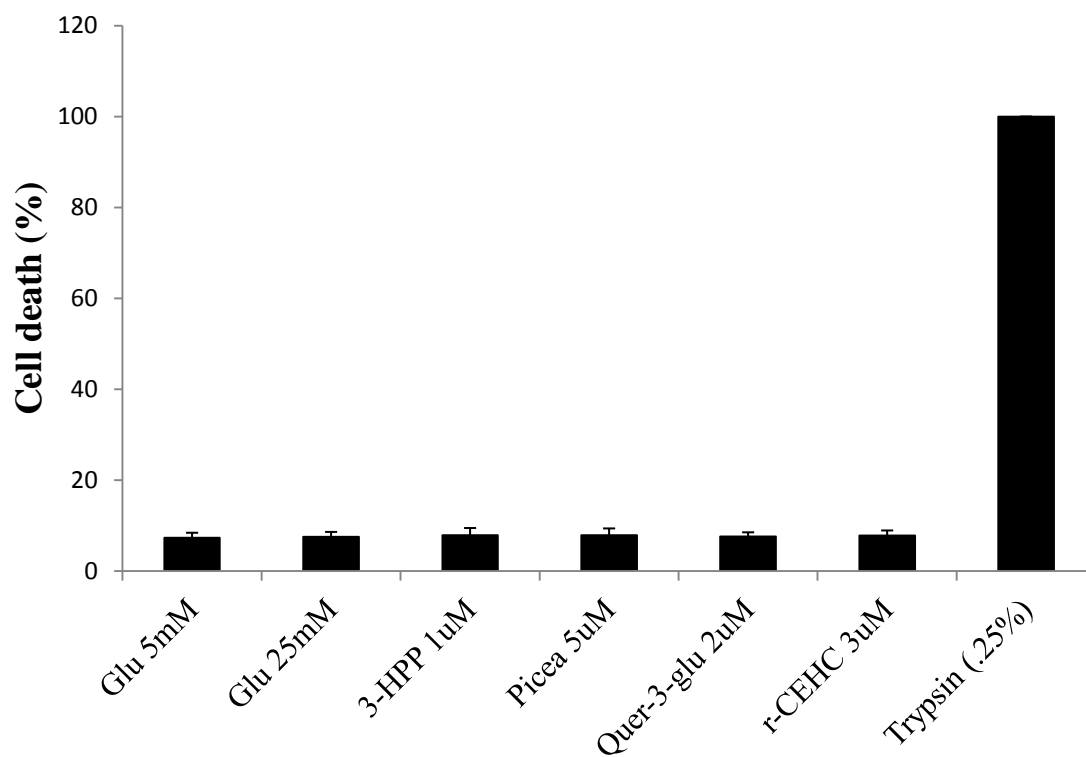


Figure 6. Cell viability during experiments. Cell viability was assessed in HAECs treated with the reagents as shown. Trypsin (.25%) served as the positive control. Results represent mean \pm SE from $n = 4$ separate experiments.

DISCUSSION

Abnormal vascular endothelial function, as defined by measures of NO bioavailability, is well described in patients with type 1 and 2 diabetes and in cardiovascular disorders that are associated with insulin-resistance, including hypertension and atherosclerosis (3-5). Hyperglycemia is an obvious candidate contributor in the development of endothelial dysfunction in association with insulin resistance. The mechanisms underlying this phenomenon have been widely investigated and are likely to be multifactorial (7-10, 14). A great deal of evidence demonstrates that grape seed extract, resveratrol, quercetin, and α -T can improve endothelial dysfunction and maintain normal cardiovascular function (15, 22-24, 37). However, these substances do not predominantly exist in the human plasma. After ingestion, they rapidly metabolized to their physiological metabolites, but there is little research that has examined whether or not these metabolites exert protective effects on endothelial cell. This led us to determine the effects of these metabolites on insulin-stimulated NO production impaired by hyperglycemia in HAECs.

Culture of HAECs in 22.2mM glucose for 5 days was previously reported to stimulate basal NO production (45). In contrast, other groups have demonstrated reduced basal NO production in response to high glucose in human coronary artery endothelial cells and BAECs (46, 47). In the present study, we could not demonstrate any effect of culture glucose concentration on basal NO production in HAECs, which is consistent

with the study of Salt et al. (48) . These differences may be due to the different endothelial cell types used or the varied concentration and duration of hyperglycemia applied.

In this study, we have demonstrated that insulin-stimulated NO production was increased in HAECs treated with 5mM glucose, but abolished in HAECs treated with 25mM glucose vs. 5mM glucose. However, this insulin-stimulated NO production was restored in HAECs by pretreated with 3-HPP (1 μ M), Picea (5 μ M), Quer-3-glu (2 μ M), and γ -CEHC (3 μ M), respectively. To our knowledge, this is the first study to indicate these metabolites rescue insulin-stimulated NO production abrogated by high glucose in HAECs.

In recent years, many studies indicated that multisite phosphorylation of specific serine (Ser) or/and threonine (Thr) residues has been involved in regulation of eNOS activity, and consequently, NO production (49, 50). We therefore hypothesized that this stimulation, inhibition, or restoration of insulin-stimulated NO production was a consequence of increased or decreased phosphorylation of kinases downstream of the insulin receptor. In this study, we found that insulin-stimulated phosphorylation of eNOS at Ser¹¹⁷⁷ and Akt at Ser⁴⁷³ was increased in HAECs treated with 5mM glucose, abolished in HAECs treated with 25mM glucose vs. 5mM glucose, and again, restored by pretreated with 3-HPP (1 μ M), Picea (5 μ M), Quer-3-glu (2 μ M), and γ -CEHC (3 μ M), respectively. However, there was no difference in basal phosphorylation of eNOS^{Ser117}, Akt^{Ser473}, ERK^{Thr202/204}, or insulin-stimulated ERK^{Thr202/204} phosphorylation in cells treated with 5mM glucose or 25mM glucose. Basal levels of those proteins were also similar when cells were pretreated with metabolites. These data suggest that the regulation of

eNOS is through Akt-mediated phosphorylation in HAECs. Normally, when insulin presents in the blood, it binds to the insulin receptor and phosphorylates the receptor, then triggers signaling cascade and mediates the downstream factors. However, insulin-resistance occurs during hyperglycemia, which desensitizes insulin receptors, and therefore, blocks insulin dependent signaling. In our experiments, 25mM glucose resulted in insulin-resistance in HAECs. Therefore, insulin-stimulated signaling through phosphorylating Ak at Ser⁴⁷³ was interrupted, and subsequently, impaired phosphorylation of eNOS at Ser¹¹⁷⁷. However, 3-HPP, Picea, Quer-3-glu, or γ -CEHC reversed the negative effect caused by high glucose.

ROS are attractive candidates as mediators of endothelial dysfunction in diabetic rats and in diabetic hypertensive patients (6, 7). Monocytes from patients with diabetes showed an increase in production of superoxide relative to those from normal controls (7). Increased lipid peroxidation and reduced antioxidant defenses have been demonstrated in patients with either type 1 or type 2 diabetes (8). In addition, several in vitro studies showed that endothelial cells grown under hyperglycemic concentration of glucose (25mM) increases the formation of ROS (51). In the current study, our finding is consistent with the previously reports. Further, our data demonstrate that high glucose-stimulated total ROS/RNS production inhibited by pretreated with 3-HPP (1 μ M), Picea (5 μ M), Quer-3-glu (2 μ M), and γ -CEHC (3 μ M), respectively. Although the mechanism regulating the decrease in ROS/RNS production by these metabolites was not investigated in our study, we speculate it may be due to their antioxidant characteristics. It is known that high glucose creates ROS by activation of oxidase enzymes, such as xanthine oxidase and NADPH oxidase (52). Moreover, eNOS itself can produce ROS due

to uncoupling when there is increased BH4 oxidation caused by high glucose (7, 10). Therefore, ROS production induced by high glucose may have been abolished due to antioxidant properties of these metabolites, such as inhibiting oxidase enzyme activities, scavenging free radicals, and reducing BH4 oxidation.

The bioavailability and metabolism of polyphenols are very complicated, and need to be clearly resolved in order to better understand their biological role and mechanisms of action. After ingestion, polyphenols are rapidly metabolized into glucuronated, methylated, or thiol conjugates in the liver, and then circulate in the blood stream. These metabolite forms are the dominant forms that are found in the blood. A limitation of many previous *in vivo* studies is that they do not account for the fact that these compounds are quickly metabolized prior to and after absorption in the gut. For example, quercetin 3-glucuronide (2 μ M) is present in plasma of human subjects 1.5h after consumption of onions (a rich source of quercetin) (43). Given such data, one can question the traditional approach of using a parent compounds to investigate and explain the mechanism of action in humans. Therefore, a strength of the present study is that we have used the metabolite forms, rather than parent compounds to investigate the mechanism responsible for improvement of high glucose-induced endothelial dysfunction.

In summary, we have shown that dominant metabolites of grape seed extract, resveratrol, quercetin, and vitamin E, respectively, restore insulin-stimulated NO production abolished by high glucose in HAECs, at their physiological concentration or 5-fold greater. This effect may be due to increasing insulin-stimulated Akt-mediated phosphorylation of eNOS at Ser¹¹⁷⁷. Additionally, at the same concentration, these metabolites can also reduce high glucose-induced ROS/RNS production in HAECs.

Future studies are required to examine whether there will be some other factors involved in this insulin signaling pathway, as well as effects of parent compounds of these metabolites side by side on high glucose-induced endothelial dysfunction in HAECs. That will help us to further understand the role of these metabolites and find out the similar and different effects between the natural and metabolite forms.

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